CLAIMS

WHAT IS CLAIMED IS:

1. A eukaryotic cell comprising an orthogonal aminoacyl-tRNA synthetase (O-RS), wherein the O-RS preferentially aminoacylates an orthogonal tRNA (O-tRNA) with at least one unnatural amino acid in the eukaryotic cell.

2. The cell of claim 1, wherein the O-RS aminoacylates the O-tRNA with the at least one unnatural amino acid at least 50% as efficiently as does an O-RS having an amino acid sequence as set forth in SEQ ID NO.: 86.

3. The cell of claim 1, wherein the at least one unnatural amino acid comprises two or more unnatural amino acids.

4. The cell of claim 1, wherein the O-RS, or a portion thereof, is encoded by a polynucleotide sequence as set forth in any one of SEQ ID NO.: 3-19, or a complementary polynucleotide sequence thereof.

5. The cell of claim 1, wherein the O-RS comprises an amino acid sequence as set forth in any one of SEQ ID NO.: 36-47, or 86, or a conservative variation thereof.

6. The cell of claim 1, wherein the O-RS aminoacylates the O-tRNA with the at least one unnatural amino acid at least 10-fold more efficiently than the O-RS aminoacylates the O-tRNA with a natural amino acid.

7. The cell of claim 1, wherein the O-RS comprises an amino acid sequence that is at least 90% identical to that of a naturally occurring tyrosyl aminoacyl-tRNA synthetase (TyrRS) and comprises two or more amino acids selected from the group consisting of:

A) valine, isoleucine, leucine, or threonine at a position corresponding to Tyr37 of *E. coli* TyrRS;

B) threonine, serine, arginine, or glycine at a position corresponding to Asp182 of *E. coli* TyrRS;

D) methionine, or tyrosine at a position corresponding to Phe183 of *E. coli* TyrRS; and,

E) serine, or alanine at a position corresponding to Leu186 of *E. coli* TyrRS.

8. The cell of claim 1, wherein the O-RS is derived from a non-eukaryotic organism.

9. The cell of claim 8, wherein the non-eukaryotic organism is *Escherichia coli,* or *Bacillus stearothermophilus.*

10. The cell of claim 1, wherein the eukaryotic cell is a yeast cell, a mammalian cell, a plant cell, an algae cell, a fungal cell, or an insect cell.

11. The cell of claim 10, wherein the eukaryotic cell is a *Saccharomyces cerevisiae* cell.

12. The cell of claim 1, wherein the O-RS has one or more improved or enhanced enzymatic properties for the at least one unnatural amino acid as compared to a natural amino acid, which properties are selected from the group consisting of: higher K_m, lower K_m, higher k_{cat}, lower k_{cat}, lower k_{cat}/k_m, and higher k_{cat}/k_m.

13. The cell of claim 1, wherein the at least one unnatural amino acid is selected from the group consisting of: a *p*-acetyl-L-phenylalanine, a *p*-iodo-L-phenylalanine, an O-methyl-L-tyrosine, a *p*-propargyloxyphenylalanine, an L-3-(2-naphthyl)alanine, a 3-methyl-phenylalanine, an O-4-allyl-L-tyrosine, a 4-propyl-L-tyrosine, a tri-O-acetyl-GlcNAcβ-serine, an L-Dopa, a fluorinated phenylalanine, an isopropyl-L-phenylalanine, a *p*-azido-L-phenylalanine, a *p*-acyl-L-phenylalanine, a *p*-benzoyl-L-phenylalanine, an L-phosphoserine, a phosphonoserine, a phosphonotyrosine, a *p*-bromophenylalanine, a *p*-amino-L-phenylalanine, an isopropyl-L-phenylalanine, an unnatural analogue of a tyrosine amino acid; an unnatural analogue of a glutamine amino acid; an unnatural analogue of a phenylalanine amino acid; an unnatural analogue of a serine amino acid; an unnatural analogue of a threonine amino acid; an alkyl, aryl, acyl, azido, cyano, halo, hydrazine, hydrazide, hydroxyl, alkenyl, alkynl, ether, thiol, sulfonyl, seleno, ester, thioacid, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, hydroxylamine, keto, or amino substituted amino acid, or any combination thereof; an amino acid with a photoactivatable cross-linker; a spin-labeled amino acid; a fluorescent amino acid; a metal binding amino acid; a metal-containing amino acid; a radioactive amino acid; a photocaged and/or photoisomerizable amino acid; a biotin or biotin-analogue containing amino acid; a keto containing amino acid; an amino acid comprising polyethylene glycol or polyether; a heavy atom substituted amino acid; a chemically cleavable or photocleavable amino acid; an amino acid with an elongated side chain; an

amino acid containing a toxic group; a sugar substituted amino acid; a carbon-linked sugar-containing amino acid; a redox-active amino acid; an α-hydroxy containing acid; an amino thio acid; an α,α disubstituted amino acid; a β-amino acid; a cyclic amino acid other than proline or histidine, and an aromatic amino acid other than phenylalanine, tyrosine or tryptophan.

14. The cell of claim 1, further comprising the at least one unnatural amino acid.

15. The cell of claim 1, further comprising an orthogonal tRNA (O-tRNA), wherein the O-tRNA recognizes a selector codon and is preferentially aminoacylated with the at least one unnatural amino acid by the O-RS.

16. The cell of claim 15, wherein the O-tRNA is derived from a non-eukaryotic organism.

17. The cell of claim 16, wherein the non-eukaryotic organism is *Escherichia coli,* or *Bacillus stearothermophilus.*

18. The cell of claim 15, wherein the O-tRNA mediates the incorporation of the at least one unnatural amino acid into a protein with at least 50% the efficiency of a tRNA produced by cellular processing of a nucleic acid that comprises a polynucleotide sequence as set forth in SEQ ID NO.: 65.

19. The cell of claim 15, wherein the O-tRNA is produced in a cell by cellular processing of a nucleic acid corresponding to SEQ ID NO.:65, and the O-RS comprises a polypeptide sequence selected from the group consisting of: SEQ ID NO.: 36-47, 86, and a conservative variation thereof.

20. The cell of claim 15, further comprising a nucleic acid that comprises a polynucleotide that encodes a polypeptide of interest, wherein the polynucleotide comprises a selector codon that is recognized by the O-tRNA.

21. The cell of claim 20, wherein the yield of the polypeptide of interest comprising the at least one unnatural amino acid is at least 5% of that obtained for the naturally occurring polypeptide of interest from a cell in which the polynucleotide lacks the selector codon.

22. The cell of claim 20, wherein the cell produces the polypeptide of interest in the absence of the at least one unnatural amino acid with a yield that is less than 30% of the yield of the polypeptide in the presence of the at least one unnatural amino acid.

23. The cell of claim 22, wherein the cell produces the polypeptide of interest in the absence of the at least one unnatural amino acid with a yield that is less than 5% of the yield of the polypeptide in the presence of the at least one unnatural amino acid.

24. The cell of claim 20, wherein the polypeptide of interest is a therapeutic protein, a diagnostic protein, an industrial enzyme, or portion thereof.

25. The cell of claim 24, wherein the polypeptide of interest comprises a protein or a portion of a protein selected from the group consisting of: a cytokine, a growth factor, a growth factor receptor, an interferon, an interleukin, an inflammatory molecule, an oncogene product, a peptide hormone, a signal transduction molecule, a steroid hormone receptor, erythropoietin (EPO), insulin, human growth hormone, an Alpha-1 antitrypsin, an Angiostatin, an Antihemolytic factor, an antibody, an Apolipoprotein, an Apoprotein, an Atrial natriuretic factor, an Atrial natriuretic polypeptide, an Atrial peptide, a C-X-C chemokine, T39765, NAP-2, ENA-78, a Gro-a, a Gro-b, a Gro-c, an IP-10, a GCP-2, an NAP-4, an SDF-1, a PF4, a MIG, a Calcitonin, a c-kit ligand, a cytokine, a CC chemokine, a Monocyte chemoattractant protein-1, a Monocyte chemoattractant protein-2, a Monocyte chemoattractant protein-3, a Monocyte inflammatory protein-1 alpha, a Monocyte inflammatory protein-1 beta, RANTES, I309, R83915, R91733, HCC1, T58847, D31065, T64262, a CD40, a CD40 ligand, a C-kit Ligand, a Collagen, a Colony stimulating factor (CSF), a Complement factor 5a, a Complement inhibitor, a Complement receptor 1, a cytokine, DHFR, an epithelial Neutrophil Activating Peptide-78, a GROα/MGSA, a GROβ, a GROγ a MIP-1α, a MIP-1δ, a MCP-1, an Epidermal Growth Factor (EGF), an epithelial Neutrophil Activating Peptide, an Erythropoietin (EPO), an Exfoliating toxin, a Factor IX, a Factor VII, a Factor VIII, a Factor X, a Fibroblast Growth Factor (FGF), a Fibrinogen, a Fibronectin, a G-CSF, a GM-CSF, a Glucocerebrosidase, a Gonadotropin, a growth factor, a growth factor receptor, a Hedgehog protein, a Hemoglobin, a Hepatocyte Growth Factor (HGF), a Hirudin, a Human serum albumin, an ICAM-1, an ICAM-1 receptor, an LFA-1, an LFA-1 receptor, an Insulin, an Insulin-like Growth Factor (IGF), an IGF-I, an IGF-II, an interferon, an IFN-α, an IFN-β, an IFN-γ, an interleukin, an IL-1, an IL-2, an IL-3, an IL-4, an IL-5, an IL-6, an IL-7, an IL-8, an IL-9, an IL-10, an IL-11, an IL-12, a Keratinocyte

Growth Factor (KGF), a Lactoferrin, a leukemia inhibitory factor, a Luciferase, a Neurturin, a Neutrophil inhibitory factor (NIF), an oncostatin M, an Osteogenic protein, an oncogene product, a Parathyroid hormone, a PD-ECSF, a PDGF, a peptide hormone, a Human Growth Hormone, a Pleiotropin, a Protein A, a Protein G, a Pyrogenic exotoxins A, B, or C, a Relaxin, a Renin, an SCF, a Soluble complement receptor I, a Soluble I-CAM 1, a Soluble interleukin receptor, a Soluble TNF receptor, a Somatomedin, a Somatostatin, a Somatotropin, a Streptokinase, a Superantigen, a Staphylococcal enterotoxins, an SEA, an SEB, an SEC1, an SEC2, an SEC3, an SED, an SEE, a steroid hormone receptor, a Superoxide dismutase (SOD), a Toxic shock syndrome toxin, a Thymosin alpha 1, a Tissue plasminogen activator, a tumor growth factor (TGF), a TGF-α, a TGF-β, a Tumor Necrosis Factor, a Tumor Necrosis Factor alpha, a Tumor necrosis factor beta, a Tumor necrosis factor receptor (TNFR), a VLA-4 protein, a VCAM-1 protein, a Vascular Endothelial Growth Factor (VEGEF), a Urokinase, a Mos, a Ras, a Raf, a Met; a p53, a Tat, a Fos, a Myc, a Jun, a Myb, a Rel, an estrogen receptor, a progesterone receptor, a testosterone receptor, an aldosterone receptor, an LDL receptor, a SCF/c-Kit, a CD40L/CD40, a VLA-4/VCAM-1, an ICAM-1/LFA-1, a hyalurin/CD44, and a corticosterone.

26. The cell of claim 20, further comprising the polypeptide of interest, or portion thereof, encoded by the nucleic acid.

27. The cell of claim 20, wherein the polypeptide of interest comprises a transcriptional modulator protein, or a portion thereof.

28. The cell of claim 27, wherein the transcription modulator protein is a transcriptional activator protein.

29. The cell of claim 28, wherein the transcriptional activator protein is GAL4.

30. The cell of claim 27, wherein the transcription modulator protein is a transcriptional repressor protein.

31. A eukaryotic cell comprising an orthogonal aminoacyl-tRNA synthetase (O-RS), an orthogonal tRNA (O-tRNA), an unnatural amino acid, and a nucleic acid that comprises a polynucleotide that encodes a polypeptide of interest, wherein the polynucleotide comprises a selector codon that is recognized by the O-tRNA, wherein the O-RS preferentially aminoacylates the orthogonal tRNA (O-tRNA) with the unnatural amino acid in the eukaryotic cell, and wherein the cell produces the polypeptide of interest

in the absence of the unnatural amino acid with a yield that is less than 30% of the yield of the polypeptide in the presence of the unnatural amino acid.

32. A eukaryotic cell comprising an orthogonal tRNA (O-tRNA), wherein the O-tRNA mediates incorporation of an unnatural amino acid into a protein that is encoded by a polynucleotide that comprises a selector codon that is recognized by the O-tRNA in vivo.

33. The cell of claim 32, wherein the O-tRNA mediates the incorporation of the unnatural amino acid into the protein at least 50% as efficiently as a tRNA that produced by cellular processing of a polynucleotide comprising a sequence as set forth in SEQ ID NO.: 65.

34. The cell of claim 32, wherein the O-tRNA comprises a polynucleotide sequence as set forth in SEQ ID NO.: 65, a cellularly processed form thereof, or a conservative variation thereof.

35. The cell of claim 32, wherein the O-tRNA is post-transcriptionally modified.

36. A nucleic acid that encodes an O-tRNA of claim 32, or a complementary polynucleotide thereof.

37. The nucleic acid of claim 36, wherein the nucleic acid comprises an A box and a B box.

38. A composition comprising a GAL4 protein, or portion thereof, in a eukaryotic cell, wherein the GAL4 protein, or portion thereof, comprises at least one unnatural amino acid.

39. A composition comprising a protein, wherein the protein comprises at least one unnatural amino acid comprising at least one post-translational modification, wherein the at least one post-translational modification comprises a saccharide moiety.

40. The composition of claim 39, wherein the at least one unnatural amino acid is a keto unnatural amino acid.

41. The composition of claim 39, wherein the at least one post-translational modification is made in vivo in a eukaryotic cell.

42. A composition comprising a protein, wherein the protein comprises at least one unnatural amino acid and at least one post-translational modification that is made in

vivo by a eukaryotic cell, wherein the post-translational modification is not naturally made by a prokaryotic cell.

43. The composition of claim 42, wherein the post-translational modification comprises attachment of an oligosaccharide to an asparagine by a GlcNAc-asparagine linkage.

44. The composition of claim 43, wherein the oligosaccharide comprises $(GlcNAc\text{-}Man)_2\text{-}Man\text{-}GlcNAc\text{-}GlcNAc$.

45. The composition of claim 42, wherein the post-translational modification comprises attachment of an oligosaccharide to a serine or threonine by a GalNAc-serine, a GalNAc-threonine, a GlcNAc-serine, or a GlcNAc-threonine linkage.

46. The composition of claim 45, wherein the oligosaccharide comprises Gal-GalNAc or Gal-GlcNAc.

47. The composition of claim 42, wherein the post-translational modification is selected from the group consisting of: acetylation, acylation, lipid-modification, palmitoylation, palmitate addition, phosphorylation, and glycolipid-linkage modification.

48. The composition of claim 42, wherein the protein comprises an amino acid sequence that is at least 75% identical to that of a therapeutic protein, a diagnostic protein, an industrial enzyme, or portion thereof.

49. The composition of claim 48, wherein at least one, but fewer than all, of a particular amino acid present in a naturally occurring version of the protein is substituted with an unnatural amino acid.

50. The composition of claim 42, wherein the protein comprises at least two unnatural amino acids.

51. The composition of claim 50, wherein the protein comprises at least two different unnatural amino acids.

52. The composition of claim 42, wherein the protein comprises at least three unnatural amino acids.

53. The composition of claim 42, wherein the protein comprises four or more unnatural amino acids.

54. The composition of claim 42, wherein the composition further comprises a pharmaceutically acceptable excipient.

55. The composition of claim 42, wherein the composition comprises at least 100 micrograms of the protein.

56. The composition of claim 42, wherein the composition comprises at least 50 μg/liter of the protein.

57. The composition of claim 42, wherein the protein comprises a secretion or localization sequence, an epitope tag, a FLAG tag, a polyhistidine tag, or a GST fusion.

58. A polypeptide selected from the group consisting of:

(a) a polypeptide that comprises an amino acid sequence as shown in any one of SEQ ID NO.: 36-47, or 86;

(b) a polypeptide that comprises an amino acid sequence encoded by a polynucleotide sequence as shown in any one of SEQ ID NO.: 3-19;

(c) a polypeptide that is specifically immunoreactive with an antibody specific for a polypeptide of (a), or (b);

(d) a polypeptide that comprises an amino acid sequence that is at least 90% identical to that of a naturally occurring tyrosyl aminoacyl-tRNA synthetase (TyrRS) and comprises two or more amino acids selected from the group consisting of: valine, isoleucine, leucine, or threonine at a position corresponding to Tyr37 of *E. coli* TyrRS; threonine, serine, arginine, or glycine at a position corresponding to Asp182 of *E. coli* TyrRS; methionine, or tyrosine at a position corresponding to Phe183 of *E. coli* TyrRS; and, serine, or alanine at a position corresponding to Leu186 of *E. coli* TyrRS;

(e) a polypeptide that comprises at least 20 contiguous amino acids of SEQ ID NO.: 36-47, or 86, and two or more amino acid substitutions selected from the group consisting of: valine, isoleucine, leucine, or threonine at a position corresponding to Tyr37 of *E. coli* TyrRS; threonine, serine, arginine, or glycine at a position corresponding to Asp182 of *E. coli* TyrRS; methionine, or tyrosine at a position corresponding to Phe183 of *E. coli* TyrRS; and, serine, or alanine at a position corresponding to Leu186 of *E. coli* TyrRS, wherein numbering of the amino acids corresponds to that of *E. coli* TyrRS; and,

(f) an amino acid sequence comprising a conservative variation of (a), (b), (c), (d), or (e).

59. A composition comprising the polypeptide of claim 58 and an excipient.

60. An antibody or antisera specifically immunoreactive with the polypeptide of claim 58.

61. A polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising a nucleotide sequence as set forth in any one of SEQ ID NO.: 3-19, or 64-85;

(b) a polynucleotide that is complementary to or that encodes a polynucleotide sequence of (a);

(c) a polynucleotide encoding a polypeptide that comprises an amino acid sequence as set forth in any one of SEQ ID NO.: 36-47, or 86, or a conservative variation thereof;

(d) a polynucleotide that encodes a polypeptide of claim 58;

(e) a nucleic acid that hybridizes to a polynucleotide of (a), (b), (c), or (d) under highly stringent conditions over substantially the entire length of the nucleic acid;

(f), a polynucleotide that encodes a polypeptide that comprises an amino acid sequence that is at least 90% identical to that of a naturally occurring tyrosyl aminoacyl-tRNA synthetase (TyrRS) and comprises two or more mutations selected from the group consisting of: valine, isoleucine, leucine, or threonine at a position corresponding to Tyr37 of *E. coli* TyrRS; threonine, serine, arginine, or glycine at a position corresponding to Asp182 of *E. coli* TyrRS; methionine, or tyrosine at a position corresponding to Phe183 of *E. coli* TyrRS; and, serine, or alanine at a position corresponding to Leu186 of *E. coli* TyrRS;

(g) a polynucleotide that is at least 98% identical to a polynucleotide of (a), (b), (c), (d), (e), or (f); and,

(h) a polynucleotide comprising a conservative variation of (a), (b), (c), (d), (e), (f), or (g).

62. A vector comprising a polynucleotide of claim 61.

63. The vector of claim 62, wherein the vector comprises a plasmid, a cosmid, a phage, or a virus.

64. The vector of claim 62, wherein the vector is an expression vector.

65. A cell comprising the vector of claim 62.

66. A method of producing an orthogonal aminoacyl-tRNA synthetase (O-RS) that preferentially aminoacylates an orthogonal tRNA with an unnatural amino acid in a eukaryotic cell, the method comprising:

(a) subjecting to positive selection, in the presence of an unnatural amino acid, a population of eukaryotic cells of a first species, wherein the eukaryotic cells each comprise: i) a member of a library of aminoacyl-tRNA synthetases (RSs), ii) an orthogonal tRNA (O-tRNA), iii) a polynucleotide that encodes a positive selection marker, and iv) a polynucleotide that encodes a negative selection marker; wherein cells that survive the positive selection comprise an active RS that aminoacylates the orthogonal tRNA (O-tRNA) in the presence of an unnatural amino acid; and,

(b) subjecting the cells that survive the positive selection to negative selection in the absence of the unnatural amino acid to eliminate active RSs that aminoacylate the O-tRNA with a natural amino acid, thereby providing the O-RS that preferentially aminoacylates the O-tRNA with the unnatural amino acid.

67. The method of claim 66, wherein the polynucleotide that encodes the positive selection marker is operably linked to a response element and the cells further comprise a polynucleotide that: a) encodes a transcriptional modulator protein that modulates transcription from the response element, and b) comprises at least one selector codon;

wherein incorporation of the unnatural amino acid into the transcriptional modulator protein by the O-tRNA aminoacylated with the unnatural amino acid results in transcription of the positive selection marker.

68. The method of claim 67, wherein the transcriptional modulator protein is a eukaryotic transcriptional modulator protein.

69. The method of claim 67, wherein the transcriptional modulator protein is a transcriptional activator protein and the selector codon is an amber stop codon.

70. The method of claim 69, wherein the amber stop codon is located in or substantially near a portion of the polynucleotide that encodes a DNA binding domain of the transcriptional activator protein.

71. The method of claim 69, wherein the transcriptional activator protein is GAL4.

72. The method of claim 66, wherein the positive selection marker provides a nutritional supplement for growth and the selection is performed on a medium that lacks the nutritional supplement.

73. The method of claim 72, wherein the polynucleotide that encodes the positive selection marker is a his3, ura3, leu2, lys2, or lacZ gene.

74. The method of claim 73, wherein the his3 gene encodes an imidazole glycerol phosphate dehydratase, which dehydratase is detected by providing 3-aminotriazole (3-AT).

75. The method of claim 67, wherein the positive selection marker fluoresces or catalyzes a luminescent reaction in the presence of a suitable reactant.

76. The method of claim 75, wherein a product of the positive selection marker is detected by fluorescence-activated cell sorting (FACS) or by luminescence.

77. The method of claim 66, wherein the positive selection marker comprises an affinity based screening marker.

78. The method of claim 66, wherein the polynucleotide that encodes the positive selection marker comprises a selector codon.

79. The method of claim 67, wherein the polynucleotide that encodes the negative selection marker is operably linked to a response element from which transcription is mediated by the transcriptional modulator protein;

wherein incorporation of a natural amino acid into the transcriptional modulator protein by the O-tRNA aminoacylated with a natural amino acid results in transcription of the negative selection marker.

80. The method of claim 66, wherein the polynucleotide that encodes the negative selection marker comprises an ura3 gene and the negative selection is accomplished on a medium that comprises 5-fluroorotic acid (5-FOA).

81. The method of claim 66, wherein the medium used for negative selection comprises a selecting or screening agent that is converted to a detectable substance by the negative selection marker.

82. The method of claim 81, wherein the detectable substance is a toxic substance.

83. The method of claim 66, wherein the negative selection marker fluoresces or catalyzes a luminescent reaction in the presence of a suitable reactant.

84. The method of claim 83, wherein a product of the negative selection marker is detected by fluorescence-activated cell sorting (FACS) or by luminescence.

85. The method of claim 66, wherein the negative selection marker comprises an affinity based screening marker.

86. The method of claim 66, wherein the polynucleotide that encodes the negative selection marker comprises a selector codon.

87. The method of claim 86, wherein the selector codon comprises an amber codon, an ochre codon, or an opal stop codon.

88. The method of claim 66, wherein the same polynucleotide encodes both the positive selection marker and the negative selection marker.

89. The method of claim 66, wherein the polynucleotide that encodes the positive selection marker comprises at least two selector codons.

90. The method of claim 66, wherein the polynucleotide that encodes the negative selection marker comprises at least two selector codons.

91. The method of claim 66, wherein one or both of the polynucleotides that encodes positive selection marker and the negative selection marker each comprises at least two different selector codons.

92. The method of claim 66, wherein one or both of the polynucleotides that encodes the positive selection marker and the negative selection marker each comprises at least two of the same selector codons.

93. The method of claim 66, wherein the unnatural amino acid is selected from the group consisting of: a *p*-acetyl-L-phenylalanine, a *p*-iodo-L-phenylalanine, an O-methyl-L-tyrosine, a *p*-propargyloxyphenylalanine, an L-3-(2-naphthyl)alanine, a 3-methyl-

phenylalanine, an O-4-allyl-L-tyrosine, a 4-propyl-L-tyrosine, a tri-O-acetyl-GlcNAcβ-serine, an L-Dopa, a fluorinated phenylalanine, an isopropyl-L-phenylalanine, a *p*-azido-L-phenylalanine, a *p*-acyl-L-phenylalanine, a *p*-benzoyl-L-phenylalanine, an L-phosphoserine, a phosphonoserine, a phosphonotyrosine, a *p*-bromophenylalanine, a *p*-amino-L-phenylalanine, an isopropyl-L-phenylalanine, an unnatural analogue of a tyrosine amino acid; an unnatural analogue of a glutamine amino acid; an unnatural analogue of a phenylalanine amino acid; an unnatural analogue of a serine amino acid; an unnatural analogue of a threonine amino acid; an alkyl, aryl, acyl, azido, cyano, halo, hydrazine, hydrazide, hydroxyl, alkenyl, alkynl, ether, thiol, sulfonyl, seleno, ester, thioacid, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, hydroxylamine, keto, or amino substituted amino acid, or any combination thereof; an amino acid with a photoactivatable cross-linker; a spin-labeled amino acid; a fluorescent amino acid; a metal binding amino acid; a metal-containing amino acid; a radioactive amino acid; a photocaged and/or photoisomerizable amino acid; a biotin or biotin-analogue containing amino acid; a keto containing amino acid; an amino acid comprising polyethylene glycol or polyether; a heavy atom substituted amino acid; a chemically cleavable or photocleavable amino acid; an amino acid with an elongated side chain; an amino acid containing a toxic group; a sugar substituted amino acid; a carbon-linked sugar-containing amino acid; a redox-active amino acid; an α-hydroxy containing acid; an amino thio acid; an α,α disubstituted amino acid; a β-amino acid; a cyclic amino acid other than proline or histidine, and an aromatic amino acid other than phenylalanine, tyrosine or tryptophan.

94. The method of claim 66, wherein step (a), (b) or both (a) and (b) further comprise providing a varying amount of an inactive synthetase, wherein the varying amount provides an additional level of selection or screening stringency.

95. The method of claim 66, further comprises subjecting the O-RS that preferentially aminoacylates the O-tRNA with the unnatural amino acid to an additional positive selection round.

96. The method of claim 66, wherein step (a), (b) or both steps (a) and (b), comprise varying a selection or screening stringency.

97. The method of claim 66, wherein the library of RSs comprises RSs derived from at least one aminoacyl-tRNA synthetase (RS) from a non-eukaryotic organism.

98. The method of claim 66, wherein the library of RSs is derived from an inactive RS.

99. The method of claim 98, wherein the inactive RS is generated by mutating an active RS.

100. The method of claim 99, wherein the inactive RS comprises an amino acid binding pocket and one or more amino acids that comprise the binding pocket are substituted with one or more different amino acids.

101. The method of claim 100, wherein the substituted amino acids are substituted with alanines.

102. The method of claim 66, wherein the library of RSs comprises a library of mutant RSs.

103. The method of claim 102, further comprises performing random mutation, site-specific mutation, recombination, chimeric construction, or any combination thereof, on a nucleic acid that encodes an RS, thereby producing the library of mutant RSs.

104. The method of claim 66, wherein the method further comprises:

(c) isolating a nucleic acid that encodes the O-RS;

(d) generating from the nucleic acid a set of polynucleotides that encode mutated O-RSs; and,

(e) repeating steps (a) and/or (b) until a mutated O-RS is obtained that preferentially aminoacylates the O-tRNA with the unnatural amino acid.

105. The method of claim 104, further comprises performing steps (c)-(e) at least two times.

106. The method of claim 104, wherein step (d) comprises random mutagenesis, site-specific mutagenesis, chimeric construction, recombination or any combination thereof.

107. The O-RS produced by the method of claim 66.

108. The method of claim 66, wherein the O-tRNA is obtained by subjecting to negative selection a population of eukaryotic cells of a first species, wherein the eukaryotic cells comprise a member of a library of tRNAs, to eliminate cells that comprise a member of the library of tRNAs that is aminoacylated by an aminoacyl-tRNA synthetase (RS) that is

endogenous to the eukaryotic cells, thereby providing a pool of tRNAs that are orthogonal to the eukaryotic cell of the first species.

109. The method of claim 108, wherein the library of tRNAs comprises tRNAs derived from at least one tRNA from a non-eukaryotic organism.

110. The method of claim 108, wherein the library of aminoacyl-tRNA synthetases (RSs) comprises RSs derived from at least one aminoacyl-tRNA synthetase (RS) from a non-eukaryotic organism.

111. The method of claim 108, wherein the library of tRNAs comprises tRNAs derived from at least one tRNA from a first non-eukaryotic organism and wherein the library of aminoacyl-tRNA synthetases (RSs) comprises RSs derived from at least one aminoacyl-tRNA synthetase (RS) from a second non-eukaryotic organism.

112. The method of claim 111, wherein the first and second non-eukaryotic organisms are the same.

113. The method of claim 111, wherein the first and second non-eukaryotic organisms are different.

114. The method of claim 108, wherein the method further comprises introducing a nucleic acid that encodes the O-tRNA and a nucleic acid that encodes the O-RS into a eukaryotic cell of a second species.

115. The method of claim 114, wherein the first species is yeast.

116. The method of claim 114, wherein the second species is selected from the group consisting of a mammal, an insect, a fungus, an algae, and a plant.

117. An O-tRNA/O-RS pair produced by the method of claim 108.

118. The method of claim 66 or 108, wherein the selecting or screening comprises one or more positive or negative selection or screening chosen from the groups consisting of: a change in amino acid permeability, a change in translation efficiency, and a change in translational fidelity, and wherein the one or more change is based upon a mutation in one or more gene in an organism in which an orthogonal tRNA-tRNA synthetase pair are used to produce protein.

119. A method of producing an orthogonal aminoacyl-tRNA synthetase (O-RS) that preferentially aminoacylates an orthogonal tRNA with an unnatural amino acid in a eukaryotic cell, the method comprising:

(a) subjecting to positive selection, in the presence of an unnatural amino acid, a population of eukaryotic cells of a first species, wherein the eukaryotic cells each comprise: i) a member of a library of aminoacyl-tRNA synthetases (RSs), ii) an orthogonal tRNA (O-tRNA), iii) a polynucleotide that encodes a positive selection marker, and iv) a polynucleotide that encodes a negative selection marker; wherein cells that survive the positive selection comprise an active RS that aminoacylates the orthogonal tRNA (O-tRNA) in the presence of an unnatural amino acid;

(b) subjecting the cells that survive the positive selection to negative selection in the absence of the unnatural amino acid to eliminate active RSs that aminoacylate the O-tRNA with a natural amino acid, thereby providing the O-RS that preferentially aminoacylates the O-tRNA with the unnatural amino acid,

wherein the O-tRNA is obtained by subjecting to negative selection a population of eukaryotic cells of a first species, wherein the eukaryotic cells comprise a member of a library of tRNAs, to eliminate cells that comprise a member of the library of tRNAs that is aminoacylated by an aminoacyl-tRNA synthetase (RS) that is endogenous to the eukaryotic cells, thereby providing a pool of tRNAs that are orthogonal to the eukaryotic cell of the first species;

(c) introducing a nucleic acid that encodes the O-tRNA and a nucleic acid that encodes the O-RS into a eukaryotic cell of a second species,

wherein the first species is yeast and the second species is selected from the group consisting of a mammal, an insect, a fungus, an algae, and a plant.

120. A method of producing in a eukaryotic cell at least one protein comprising at least one unnatural amino acid, the method comprising:

growing, in an appropriate medium, a eukaryotic cell that comprises a nucleic acid that comprises at least one selector codon and encodes the protein; wherein the medium comprises an unnatural amino acid and the eukaryotic cell comprises:

an orthogonal tRNA (O-tRNA) that functions in the cell and recognizes the selector codon; and

an orthogonal aminoacyl tRNA synthetase (O-RS) that preferentially aminoacylates the O-tRNA with the unnatural amino acid.

121. The method of claim 120, wherein the O-RS aminoacylates the O-tRNA with the unnatural amino acid at least 50% as efficiently as does an O-RS having an amino acid sequence as set forth in SEQ ID NO.: 86.

122. The method of claim 120, wherein the O-tRNA comprises, is cellularly processed from, or is encoded by SEQ ID NO.: 64 or 65, or a complementary polynucleotide sequence thereof.

123. The method of claim 120, wherein the O-RS comprises SEQ ID NO.: 36-48 or 86.

124. The method of claim 120, wherein the unnatural amino acid is selected from the group consisting of: a *p*-acetyl-L-phenylalanine, a *p*-iodo-L-phenylalanine, an O-methyl-L-tyrosine, a *p*-propargyloxyphenylalanine, an L-3-(2-naphthyl)alanine, a 3-methyl-phenylalanine, an O-4-allyl-L-tyrosine, a 4-propyl-L-tyrosine, a tri-O-acetyl-GlcNAcβ-serine, an L-Dopa, a fluorinated phenylalanine, an isopropyl-L-phenylalanine, a *p*-azido-L-phenylalanine, a *p*-acyl-L-phenylalanine, a *p*-benzoyl-L-phenylalanine, an L-phosphoserine, a phosphonoserine, a phosphonotyrosine, a *p*-bromophenylalanine, a *p*-amino-L-phenylalanine, an isopropyl-L-phenylalanine, an unnatural analogue of a tyrosine amino acid; an unnatural analogue of a glutamine amino acid; an unnatural analogue of a phenylalanine amino acid; an unnatural analogue of a serine amino acid; an unnatural analogue of a threonine amino acid; an alkyl, aryl, acyl, azido, cyano, halo, hydrazine, hydrazide, hydroxyl, alkenyl, alkynl, ether, thiol, sulfonyl, seleno, ester, thioacid, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, hydroxylamine, keto, or amino substituted amino acid, or any combination thereof; an amino acid with a photoactivatable cross-linker; a spin-labeled amino acid; a fluorescent amino acid; a metal binding amino acid; a metal-containing amino acid; a radioactive amino acid; a photocaged and/or photoisomerizable amino acid; a biotin or biotin-analogue containing amino acid; a keto containing amino acid; an amino acid comprising polyethylene glycol or polyether; a heavy atom substituted amino acid; a chemically cleavable or photocleavable amino acid; an amino acid with an elongated side chain; an amino acid containing a toxic group; a sugar substituted amino acid; a carbon-linked sugar-containing amino acid; a redox-active amino acid; an α-hydroxy containing acid; an amino

thio acid; an α,α disubstituted amino acid; a β-amino acid; a cyclic amino acid other than proline or histidine, and an aromatic amino acid other than phenylalanine, tyrosine or tryptophan.

125. The method of claim 120, wherein the protein comprises a therapeutic protein, a diagnostic protein, an industrial enzyme, or portion thereof.

126. The method of claim 120, wherein the protein comprises a protein or a portion of a protein selected from the group consisting of: a cytokine, a growth factor, a growth factor receptor, an interferon, an interleukin, an inflammatory molecule, an oncogene product, a peptide hormone, a signal transduction molecule, a steroid hormone receptor, erythropoietin (EPO), insulin, human growth hormone, an Alpha-1 antitrypsin, an Angiostatin, an Antihemolytic factor, an antibody, an Apolipoprotein, an Apoprotein, an Atrial natriuretic factor, an Atrial natriuretic polypeptide, an Atrial peptide, a C-X-C chemokine, T39765, NAP-2, ENA-78, a Gro-a, a Gro-b, a Gro-c, an IP-10, a GCP-2, an NAP-4, an SDF-1, a PF4, a MIG, a Calcitonin, a c-kit ligand, a cytokine, a CC chemokine, a Monocyte chemoattractant protein-1, a Monocyte chemoattractant protein-2, a Monocyte chemoattractant protein-3, a Monocyte inflammatory protein-1 alpha, a Monocyte inflammatory protein-1 beta, RANTES, I309, R83915, R91733, HCC1, T58847, D31065, T64262, a CD40, a CD40 ligand, a C-kit Ligand, a Collagen, a Colony stimulating factor (CSF), a Complement factor 5a, a Complement inhibitor, a Complement receptor 1, a cytokine, DHFR, an epithelial Neutrophil Activating Peptide-78, a GROα/MGSA, a GROβ, a GROγ a MIP-1α, a MIP-1δ, a MCP-1, an Epidermal Growth Factor (EGF), an epithelial Neutrophil Activating Peptide, an Erythropoietin (EPO), an Exfoliating toxin, a Factor IX, a Factor VII, a Factor VIII, a Factor X, a Fibroblast Growth Factor (FGF), a Fibrinogen, a Fibronectin, a G-CSF, a GM-CSF, a Glucocerebrosidase, a Gonadotropin, a growth factor, a growth factor receptor, a Hedgehog protein, a Hemoglobin, a Hepatocyte Growth Factor (HGF), a Hirudin, a Human serum albumin, an ICAM-1, an ICAM-1 receptor, an LFA-1, an LFA-1 receptor, an Insulin, an Insulin-like Growth Factor (IGF), an IGF-I, an IGF-II, an interferon, an IFN-α, an IFN-β, an IFN-γ, an interleukin, an IL-1, an IL-2, an IL-3, an IL-4, an IL-5, an IL-6, an IL-7, an IL-8, an IL-9, an IL-10, an IL-11, an IL-12, a Keratinocyte Growth Factor (KGF), a Lactoferrin, a leukemia inhibitory factor, a Luciferase, a Neurturin, a Neutrophil inhibitory factor (NIF), an oncostatin M, an Osteogenic protein, an oncogene product, a Parathyroid hormone, a PD-ECSF, a PDGF, a peptide hormone, a Human

Growth Hormone, a Pleiotropin, a Protein A, a Protein G, a Pyrogenic exotoxins A, B, or C, a Relaxin, a Renin, an SCF, a Soluble complement receptor I, a Soluble I-CAM 1, a Soluble interleukin receptor, a Soluble TNF receptor, a Somatomedin, a Somatostatin, a Somatotropin, a Streptokinase, a Superantigen, a Staphylococcal enterotoxins, an SEA, an SEB, an SEC1, an SEC2, an SEC3, an SED, an SEE, a steroid hormone receptor, a Superoxide dismutase (SOD), a Toxic shock syndrome toxin, a Thymosin alpha 1, a Tissue plasminogen activator, a tumor growth factor (TGF), a TGF-α, a TGF-β, a Tumor Necrosis Factor, a Tumor Necrosis Factor alpha, a Tumor necrosis factor beta, a Tumor necrosis factor receptor (TNFR), a VLA-4 protein, a VCAM-1 protein, a Vascular Endothelial Growth Factor (VEGEF), a Urokinase, a Mos, a Ras, a Raf, a Met; a p53, a Tat, a Fos, a Myc, a Jun, a Myb, a Rel, an estrogen receptor, a progesterone receptor, a testosterone receptor, an aldosterone receptor, an LDL receptor, a SCF/c-Kit, a CD40L/CD40, a VLA-4/VCAM-1, an ICAM-1/LFA-1, a hyalurin/CD44, and a corticosterone.

127. The method of claim 120, wherein the protein comprises a transcriptional modulator protein or a portion thereof.

128. The method of claim 127, wherein the transcription modulator protein is a transcriptional activator protein.

129. The method of claim 128, wherein the transcriptional activator protein is GAL4.

130. The method of claim 127, wherein the transcription modulator protein is a transcriptional repressor protein.

131. A protein produced by the method of claim 120.

132. The protein of claim 131, wherein the protein is further modified through the unnatural amino acid.

133. The protein of claim 131, wherein the protein is modified by at least one post-translational modification in vivo and wherein the post-translational modification is selected from the group consisting of: N-glycosylation, O-glycosylation, acetylation, acylation, lipid-modification, palmitoylation, palmitate addition, phosphorylation, and glycolipid-linkage modification.

134. A method of producing a screening or selecting transcriptional modulator protein, the method comprising:

selecting a first polynucleotide sequence, wherein the polynucleotide sequence encodes a nucleic acid binding domain;

mutating the first polynucleotide sequence to include at least one selector codon, thereby providing a screening or selecting polynucleotide sequence;

selecting a second polynucleotide sequence, wherein the second polynucleotide sequence encodes a transcriptional activation domain;

providing a construct that comprises the screening or selecting polynucleotide sequence operably linked to the second polynucleotide sequence; and,

introducing the construct, an unnatural amino acid, an orthogonal tRNA synthetase (O-RS) and an orthogonal tRNA (O-tRNA) into a cell, wherein the O-RS preferentially aminoacylates the O-tRNA with the unnatural amino acid and the O-tRNA recognizes the selector codon and incorporates the unnatural amino acid into the nucleic acid binding domain, in response to the selector codon in the screening or selecting polynucleotide sequence, thereby providing the screening or selecting transcriptional modulator protein.

135. The screening or selecting transcriptional modulator protein produced by claim 134.

136. A kit for producing a protein that comprises at least one unnatural amino acid in a cell, the kit comprising: a container containing a polynucleotide sequence encoding an O-tRNA, and a polynucleotide sequence encoding an O-RS or an O-RS.

137. The kit of claim 136, wherein the kit further comprises at least one unnatural amino acid.

138. The kit of claim 136, wherein the kit further comprises instructional materials for producing the protein.